Reference: *Biol. Bull.* **179:** 201–206. (October, 1990)

Rhizophydium littoreum on the Eggs of *Cancer anthonyi:* Parasite or Saprobe?

JEFFREY D. SHIELDS

Department of Biological Sciences, and the Marine Science Institute, University of California, Santa Barbara, California, 93106

Abstract. The relationship between host and symbiont is often difficult to assess and quantify. A novel technique that may help assess the host-symbiont relationship of organisms found in crab egg masses is described. This technique may have application in determining the relationship of other host-symbiont associations. Crab eggs were killed cryogenically and exposed in combinations with live eggs to a previously unreported symbiont of crab egg masses. The results indicated that the chytrid *Rhizophydium littoreum* is primarily a saprobe that attacks dead eggs; yet at high zoospore densities, it attacks and kills live eggs. Furthermore, *R. littoreum* is the first chytridiomycete to be reported from a marine crustacean host. It was highly prevalent on the eggs of its host and was found throughout the year.

Introduction

Symbionts are, broadly speaking, two organisms living in association together (de Bary, 1879). There is a wide range of relationships that symbioses encompass—*e.g.*, mutualism, commensalism, and parasitism (Noble and Noble, 1985)—and these relationships are often difficult to define. Several symbionts live in the broods of commercially important crabs and lobsters [*e.g., Callinectes sapidus* (Rogers-Talbert, 1948), *Cancer anthonyi* (Shields *et al.*, 1990), *Cancer magister* (Fisher and Wickham, 1976; Wickham, 1986), *Homarus americanus* (Aiken *et al.*, 1985; Campbell and Brattey, 1985), *Paralithodes camtschatica* (Wickham *et al.*, 1985; Kuris *et al.*, 1991)]. Indeed, some of these symbionts are egg parasites or predators that may cause widespread brood losses in certain commercial stocks of crustaceans (Wickham, 1986; Kuris and Wickham, 1987; Kuris *et al.*, 1991). Species of bacteria, zoosporic fungi, nemerteans, and amphipods have been found together on individual crab hosts, and all have been implicated as agents that cause egg mortality. The contributions of these symbionts to egg mortality in populations of some of these decapod hosts have only recently been elucidated (Shields and Kuris, 1988; Kuris *et al.*, 1991).

This is the first report of a chytrid symbiont infesting a marine crustacean host, the yellow rock crab, *Cancer anthonyi*. The fungus-like chytrid, *Rhizophydium littoreum* Amon, 1984, was recovered and isolated from the eggs of *C. anthonyi* during a field survey for the presence of egg mass symbionts (Shields and Kuris, 1988; Shields *et al.*, 1990). The prevalence of the chytrid in the broods of *C. anthonyi* prompted an investigation into its role as a possible agent of egg mortality. The host-symbiont relationship was examined in laboratory studies that included a novel experimental protocol.

Materials and Methods

Ovigerous crabs were trapped in the Santa Barbara Channel, between Summerland and Gaviota, California. The crabs were collected at depths of 10–100 m by a commercial fisherman, transported directly to the laboratory, and maintained at ambient seawater temperatures in 280-l flow-through fiberglass aquaria.

The presence of *R. littoreum* was established as follows. The egg samples were removed to sterile petri dishes containing UV-filtered seawater (2×35 μm activated charcoal filters, one ultraviolet-light filter, Rainbow Plastics, Filter Division, El Monte, California) for direct examination

Received 12 December 1989; accepted 20 July 1990.

Present address: Department of Parasitology, University of Queensland, St. Lucia, Brisbane, 4067, Australia.

with a stereo microscope. After three days the samples were observed again and streaked with sterile pipettes onto a sterile modified Vishniac medium (MV): 1.0 g glucose, 1.0 g gelatin hydrolysate, 0.1 g bacto-peptone, 0.1 g yeast extract, 1.0 l seawater, 15.0 g agar (modified from Fuller *et al.*, 1964), containing antibiotics (500 mg each of penicillin-G and streptomycin sulfate per liter). Seawater controls were also cultured. After an additional 3–5 days the plates were examined for the presence or absence of chytrid thalli. Other substrates from the habitat of *C. anthonyi* were not examined for chytrids.

Pure cultures of *R. littoreum* were isolated from the eggs of different crabs on numerous occasions. Isolated cultures of *R. littoreum* were grown in sterile liquid MV medium (MV as above with 1.0 g agar instead of 15.0 g agar). Cultures were maintained both with, and without, antibiotics (500 mg/l each of penicillin-G and streptomycin sulfate, Sigma Co.) at 15° and 20°C.

To establish the host-symbiont relationship, live and dead crab eggs (see below) were exposed to the chytrid separately and in combination. Before exposure, egg-bearing setae were removed from the pleopod and placed in UV-filtered seawater. Samples consisting of 80–300 eggs that were attached to individual and intertwined setae were counted, and the number of dead eggs and their apparent cause of death (*e.g.*, mechanical disruption, infertility, etc.) were noted. After they had been counted, the samples were washed in UV-filtered seawater containing 1.0% bleach for 3–5 min to kill or remove microorganisms. They were then placed in 35 × 10 mm plastic petri dishes with 3.0 ml of UV-filtered seawater containing antibiotics (500 mg/l each of penicillin-G and streptomycin sulfate). Combinations of live and dead eggs (80–300 of each per replicate) were then exposed to approximately 1000 zoospores of *R. littoreum*.

Samples of egg-bearing crab setae (80–300 eggs/sample) were plunged into liquid Freon (Pelco) in a metal dish jacketed with liquid nitrogen. The eggs thus killed were thawed in ice cold seawater, and equilibrated to 15°C. The samples were then counted and the few broken eggs were recorded. The coats of the eggs killed in this manner were not grossly disrupted.

The effect of zoospore density on mortality was determined by exposure of eggs to 10, 100, and 1000 zoospores/ml at 15°C. Zoospores from cultures of *R. littoreum* were counted with the aid of a hemocytometer (Levy counting chamber). Three replicates of the culture were counted and the appropriate dilutions were made to give estimated densities of 10, 100, and 1000 zoospores/ml. From eight to ten replicates were examined in each treatment. A separate treatment of eggs exposed to antibiotics and diluted MV medium served as the control. Crab egg mortality (*i.e.*, the number of living or dead eggs attacked by the chytrid) was assessed every two to three days for ten days. Eggs in control exposures experienced negligible mortality.

Results

Rhizophydium littoreum was identified by Dr. D. J. Barr (Fig. 1). Representative specimens (Barr #580) have been deposited at the Biosystematics Research Centre (Wm. Saunders Bldg., C.E.F. Ottawa, Ontario, K1A 0C6, Canada). The monocentric thalli of *R. littoreum* ranged from 30 to 90 μm wide on crab eggs and in MV medium. Smaller immature thalli were also observed. The thallus was epibiotic and typically resided externally on the crab egg with the rhizoids penetrating through the egg coat into the contents of the egg. An apophysis was occasionally observed in MV culture. In culture, the life cycle of the chytrid took approximately 3–5 days from the zoospore stage to the production of a mature sporangium (at 15°C).

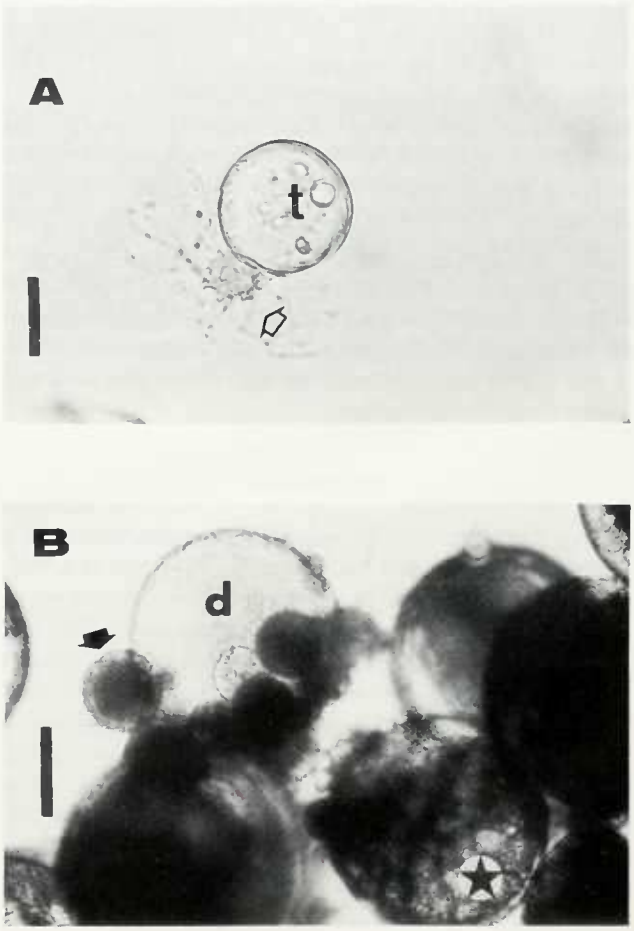

Figure 1. (A) *Rhizophydium littoreum* from culture; sporangium (t) and rhizoids (arrow); bar = 50 μm. (B) *Rhizophydium littoreum* in live eggs only treatment. Note the dead egg (d) with thallus attached (arrow), and live egg (star); bar = 100 μm.

Zoospores were posteriorly uniflagellate and ranged in size from 3 to 4 μm in diameter.

Live eggs that were successfully attacked by the chytrid lost all internal integrity over the course of 2 days at 15°C. Live eggs that were unsuccessfully attacked by the chytrid developed normally. The chytrid survived infrequently on the external coat of unsuccessfully attacked eggs. The thalli of these chytrids did not grow larger than 40 μm and did not produce zoospores. The rhizoids of these thalli were directed outward into the surrounding medium and did not appear to penetrate the egg coat.

The prevalence of *R. littoreum* from *Cancer anthonyi* was established by regular monthly or bimonthly samples of eggs taken from crabs from the Santa Barbara Channel off the coast of southern California. Prevalence ranged from 14 to 52% of the broods examined (overall prevalence = 29%, $N = 225$) throughout the year (Oct. 1985–Sept. 1986) but showed no significant seasonality ($G_H = 12$, $P < 0.10$, $df = 8$).

Rhizophydium littoreum attacked dead eggs preferentially (Figs. 2, 3). No significant differences were observed between the proportion of dead eggs attacked by the chytrid in the presence or absence of live eggs (80–300 of both live and dead eggs) (t-test between dead with live and dead only, $P < 0.05$, 7 of 7 comparisons at day 10). In the presence of live and dead eggs, the chytrid preferred dead eggs. Significantly more dead eggs were attacked by the chytrid than were live eggs (ANOVA, Bonferroni's inequality, $P < 0.05$, four comparisons/experiment), except in the EDS I treatments between live eggs and dead eggs exposed to 100 zoospores (Fig. 3B, C). It attacked and killed live eggs only at high zoospore densities (Fig. 2, 1000 zsp/ml initial exposure) or when zoospores were visible at high densities in treatments (Fig. 3A, B, typically 3–6 days after exposure). In the presence of dead eggs, significantly fewer live eggs were attacked and killed in several treatments than were live eggs from separate exposures (t-test between live with dead and live only, $P < 0.05$, 3 of 7 comparisons at day 10). No significant differences were observed in the proportion of live eggs attacked by the chytrid in 4 of 7 comparisons (at day 10) in the presence or absence of dead eggs (t-test as above, $P > 0.05$).

The stage of egg development did not influence the proportion of dead eggs attacked by the chytrid. In contrast, on live eggs, the chytrid preferred eggs in later stages of embryogenesis (Fig. 2). Significantly fewer live eggs were attacked in early stages of embryogenesis (EDS I) than in later stages (ANOVA, arcsin transformation of proportions, Sidak's inequality, $P < 0.01$), but this pattern was not consistent between experiments (*e.g.*, compare Fig. 3B, E).

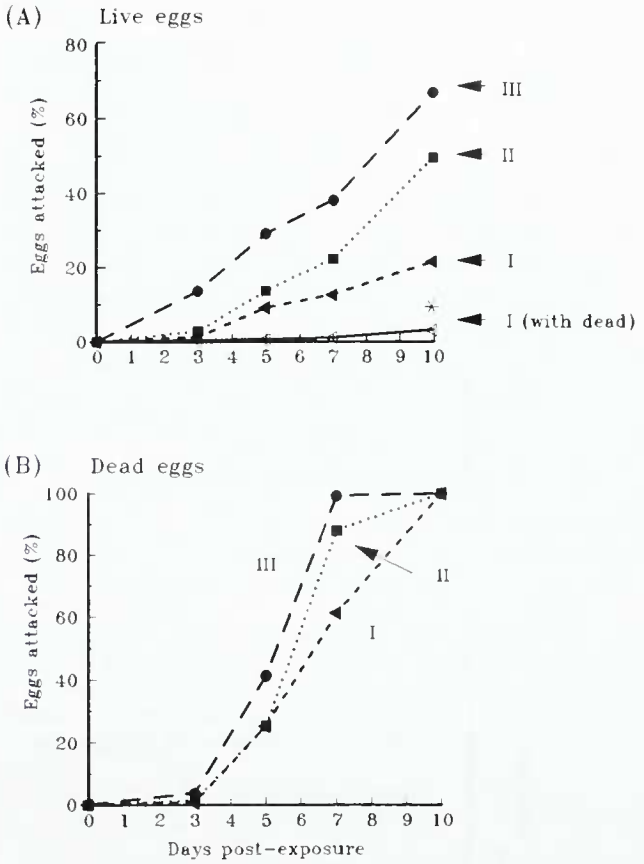

Figure 2. Infection dynamics of *Rhizophydium littoreum* on (A) live eggs and (B) dead eggs after exposure to 1000 zoospores. Live eggs in the presence of dead eggs are noted (with dead). Roman numerals refer to early (EDS I), middle (EDS II), and late (EDS III) stages in embryogenesis (Shields and Kuris, 1988; Shields *et al.*, 1990). Error bars not shown. *$P < 0.05$, significantly different from treatment with live eggs alone (t-test between EDS I live eggs alone and live eggs with dead eggs, at day 10). Not shown is the dead eggs in the presence of live eggs treatment. The data were not significantly different from the dead eggs only treatment.

Discussion

The results show conclusively that *R. littoreum* can kill live eggs, but it prefers dead eggs. Under natural conditions (*i.e.*, low zoospore density), *R. littoreum* may be a facultative parasite, but it is more likely a saprobe that lives on dead eggs. Indeed, in some cases, fewer live eggs were attacked by the chytrid when dead eggs were present than when dead eggs were absent.

Less conclusive in the laboratory was the relationship between crab embryogenesis and chytrid-related mortality. Consistent patterns were not observed (*e.g.*, Fig. 3B, E). Variations in fungal pathogenicity or host resistance/susceptibility manifested by slower growth rates of the thalli or decreased zoospore production may account for

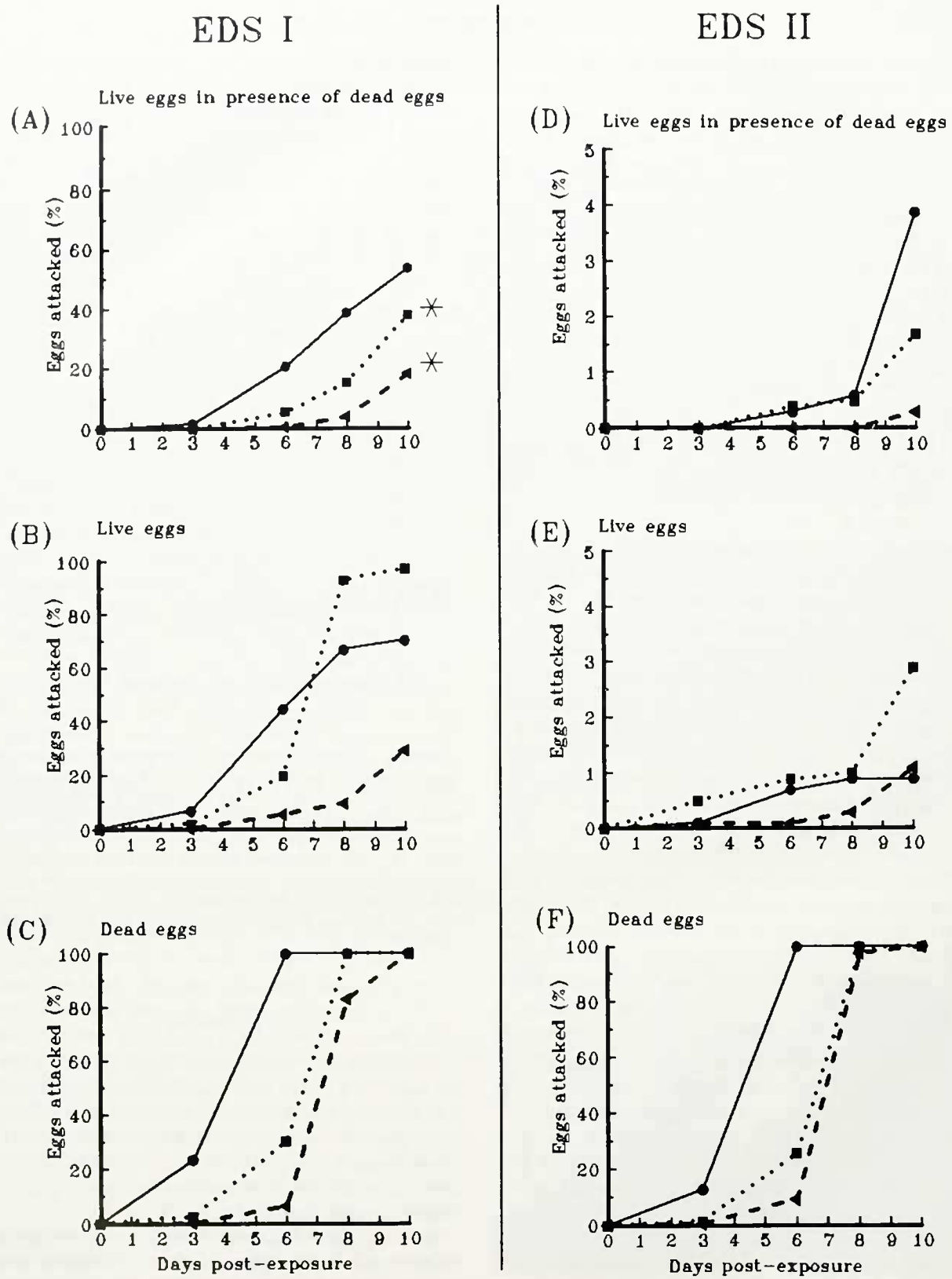

Figure 3. Infection dynamics of *Rhizophydium littoreum* on eggs of different developmental stages (EDS I or II) after exposure to different zoospore densities. Eggs were exposed to densities of 10 zoospores (triangles and dashed lines), 100 zoospores (boxes and dotted lines), and 1000 zoospores (circles and solid lines). Error bars not shown. $^*P < 0.05$, significantly different from treatments with live eggs alone (*t*-test, day 10). Not shown is the dead eggs in the presence of live eggs treatment. The data were not significantly different from the dead eggs only treatment.

the differences in attack rates of the chytrid between EDS classes. Pathogenicity and resistance have been examined in fungi-plant associations (*e.g.*, Miedaner, 1988; De Nooij and Van Damme, 1988; Alexander, 1989), but have received scant attention in marine associations. The experimental protocol can easily be manipulated to examine these factors in more detail.

Chytridiomycetes develop in a variety of live and dead fungi, algae, diatoms, and higher plants (Sparrow, 1963). They damage host cells or tissues by direct penetration of rhizoids, which in the living host results in death. Chytrids can occur as saprobes, and facultative and obligate parasites in nature (Barr, pers. comm.).

Previously, *Rhizophydium littoreum* has only been reported from the siphonous green algae, *Bryopsis plumosa* and *Codium* sp. (Kazama, 1972; Amon, 1984). It can be readily established in different culture media (Kazama, 1972; Amon, 1984, 1986). Chytrid parasites of other Metazoa have been reported from the eggs of cestodes and rotifers (Sparrow, 1963) and from aquatic copepods and ostracods (Whisler *et al.*, 1974; Weiser, 1977). A chytrid-like organism from the branchial lamellae of a shrimp (Uzmann and Haynes, 1969) may be a thraustochytrid, *Schizochytrium*.

Dead eggs are abundant in the broods of many decapod crustaceans (for review see Kuris, 1991). Indeed, populations of several commercially harvested species have recently suffered catastrophic brood losses to symbiotic agents (Wickham, 1986; Kuris *et al.*, 1991). *Cancer anthonyi*, however, experiences a relatively small degree of egg mortality (~5.0%, Shields *et al.*, 1990). This small degree of egg mortality translates into several thousand dead eggs per brood because a large (>140 mm carapace width) *Cancer anthonyi* can oviposit up to 3 million eggs (Shields, 1991). Hence, sufficient dead eggs to provide a substrate for *R. littoreum* may occur in the egg masses of the majority of the ovigerous population of *C. anthonyi*. In addition, female *Cancer* crabs bury themselves in the substrate during oviposition; their broods may become infested with the chytrid at that time.

Several species of zoosporic fungi occur on the eggs of decapods (*e.g., Atkinsiella dubia, Haliphthoros milfordensis, Lagenidium callinectes, Leptolegniella marina, Pythium thalassium*). The use of live and dead egg treatments may help to establish the role of these zoosporic fungal "pathogens." In addition, the experimental protocol may help to elucidate the roles of egg parasites in other systems.

Acknowledgments

Drs. D. J. Barr and A. Ulken identified the chytrid. Gil Crabbe collected the crabs. Drs. A. M. Kuris, I. Ross, and R. K. Zimmer-Faust provided encouragement and criticisms. Special thanks to Robert Faris, and to Drs. Page Erickson and Steven Fisher for the use of cryogenic devices. This work was funded, in part, by a patent grant from UCSB general funds and is also a result of research sponsored in part by NOAA, National Sea Grant College Program, Department of Commerce, under grant number NA80AA-D-00120, project number R/F-75 (Drs. A. M. Kuris and D. E. Wickham), through the California Sea Grant College Program, and in part by the California State Resources Agency. The U. S. Government is authorized to reproduce and distribute this article for governmental purposes.

Literature Cited

Aiken, D. E., S. L. Waddy, and L. S. Uhazy. 1985. Aspects of the biology of *Pseudocarcinonemertes homari* and its association with the American lobster, *Homarus americanus*. *Can. J. Fish. Aquat. Sci.* **42**: 351–356.

Alexander, H. M. 1989. An experimental field study of another smut disease of *Silene alba* caused by *Ustilago violacea*—genotypic variation and disease incidence. *Evolution* **43**: 835–847.

Amon, J. P. 1984. *Rhizophydium littoreum*: a chytrid from siphonaceous marine algae—an ultrastructural examination. *Mycologia* **76**: 132.

Amon, J. P. 1986. Growth of marine chytrids at ambient nutrient levels. Pp. 69–80 in *The Biology of Marine Fungi*, Cambridge University Press.

de Bary, H. A. 1879. *Die Erscheinung der Symbiose*. Strassburg, Karl J. Tübner.

Campbell, A., and J. Brattey. 1985. Egg loss from the American lobster, *Homarus americanus*, in relation to nemertean, *Pseudocarcinonemertes homari*, infestation. *Can. J. Fish. Aquat. Sci.* **43**: 772–780.

De Nooij, M. P., and J. M. M. Van Damme. 1988. Variation in host susceptibility among and within populations of *Plantago lanceolata* L. infected by the fungus *Phomopsis subordinaria* (Desm.) Trav. *Oecologia* **75**: 535–538.

Fisher, W. S., and D. E. Wickham. 1976. Mortality and epibiotic fouling of eggs from wild populations of the Dungeness crab, *Cancer magister*. *Fish. Bull. NOAA* **74**: 201–207.

Fuller, M. S., B. E. Fowles, and D. J. McLaughlin. 1964. Isolation and pure culture study of marine phycomycetes. *Mycologia* **56**: 745–756.

Kazama, F. Y. 1972. Development and morphology of a chytrid isolated from *Bryopsis plumosa*. *Can. J. Bot.* **50**: 499–505.

Kuris, A. M. 1991. A review of patterns and causes of crustacean brood mortality. In *Crustacean Issues 6, Crustacean Egg Production*, A. M. Wenner and A. M. Kuris, eds. Balkema Rotterdam. (in press).

Kuris, A. M., and D. E. Wickham. 1987. Effect of nemertean egg predators on crustaceans. *Bull. Mar. Sci.* **41**: 151–164.

Kuris, A. M., J. D. Shields, S. F. Blau, A. J. Paul and D. E. Wickham. 1991. Infestation by brood symbionts and their impact on egg mortality of the red king crab, *Paralithodes camschatica*, in Alaska: geographic and temporal variation. *Can. J. Fish. Aquat. Sci.* (in press).

Miedaner, T. 1988. The development of a host-pathogen system for evaluating *Fusarium* resistance in early growth stages of wheat. *J. Phytopathol.* (Berl.) **121**: 150–158.

Noble, E. R., and G. A. Noble. 1982. *Parasitology, The Biology of Animal Parasites*. Lea and Febiger, Philadelphia.

Rogers-Talbert, R. 1948. The fungus *Lagenidium callinectes* Couch (1942) on eggs of the blue crab in Chesapeake Bay. *Biol. Bull.* **94:** 214–228.

Shields, J. D. 1991. The reproductive ecology and fecundity of *Cancer* crabs. In *Crustacean Issues* 6, *Crustacean Egg Production*, A. M. Wenner and A. M. Kuris, eds. Balkema Rotterdam. (in press).

Shields, J. D., and A. M. Kuris. 1988. An *in vitro* analysis of egg mortality in *Cancer anthonyi*: the role of symbionts and temperature. *Biol. Bull.* **174:** 267–275.

Shields, J. D., R. K. Okazaki, and A. M. Kuris. 1990. Brood mortality and egg predation by the nemertean, *Carcinonemertes epialti*, on the yellow rock crab, *Cancer anthonyi*, in southern California. *Can. J. Fish. Aquat. Sci.* **47:** 1275–1281.

Sparrow, F. K. 1963. *Aquatic Phycomycetes*, 2nd ed., University of Michigan Press, Ann Arbor.

Uzmann, J. R., and E. B. Haynes. 1969. A mycosis of the pandalid shrimp, *Dichelopandalus leptoceros* (Smith). *J. Invertebr. Pathol.* **12:** 275–277.

Weiser, J. 1977. The crustacean intermediary host of the fungus *Coelomomyces chironomi* Rasin. *Ceska Mykol.* **31:** 81–90.

Wickham, D. E. 1986. Epizootic infestations by nemertean brood parasites on commercially important crustaceans. *Can. J. Fish. Aquat. Sci.* **43:** 2295–2302.

Wickham, D. E., S. F. Blau, and A. M. Kuris. 1985. Preliminary report on egg mortality in Alaskan king crabs caused by the egg predator *Carcinonemertes*. Pp. 265–270 in *Proceedings of the International King Crab Symp. Ser.*, Alaska Sea Grant Rep. 85-12.

Whisler, H. C., S. L. Zebold, and J. A. Shemanchuk. 1974. Alternate host for mosquito parasite *Coelomomyces*. *Nature* **251:** 715–716.